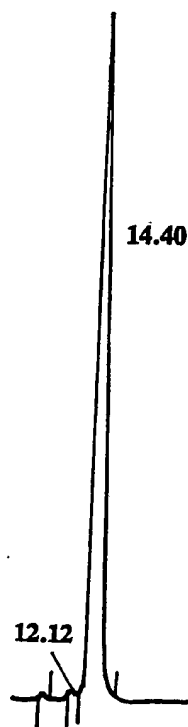




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(54) Title: STABLE DOXORUBICIN/LIPOSOME COMPOSITION**(57) Abstract**

A lyophilized doxorubicin/lipid composition which is stable against doxorubicin breakdown on storage. The composition is formed by lyophilizing a doxorubicin liposome suspension having a pH between 3.0 and 4.4.

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⁺ It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

STABLE DOXORUBICIN/LIPOSOME COMPOSITION5 1. Field of the Invention

The present invention relates to a stable, lyophilized doxorubicin/liposome composition.

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3. Background of the Invention

Doxorubicin is a potent chemotherapeutic agent effective against a broad spectrum of neoplasms (Aubel-Sadron, Young). However, the use of the drug is limited by serious side effects. Its acute toxicity includes malaise, nausea, vomiting, myelosuppression, and severe alopecia. In addition, cumulative and irreversible cardiac damage occurs with repeated administration, which seriously limits the use of the drug in protracted treatment (Young).

When administered in liposomal form, doxorubicin retains its therapeutic effectiveness against animal tumors, but is significantly less toxic (Forssen, Gabizon, 1985). The drug-protective effect of liposomes is due, at least in part, to a marked alteration in plasma pharmacokinetics and tissue disposition of the injected drug (Gabizon, 1982, 1983; Juliano).

Recently, it has been recognized that liposomal doxorubicin preparations are relatively unstable on storage in liquid form, as evidenced by rapid breakdown of both doxorubicin and liposomal lipids. The instability of doxorubicin, when combined in liposomal form, appears to involve free radical and related oxidative mechanisms, since the rate and extent of lipid and drug damage is substantially reduced by free-radical quenchers, such as alpha-tocopher-

ol, and by an iron-specific chelator, such as desferrioxamine (U.S. Patent 4,898,735).

The rate and extent of drug and lipid damage in a doxorubicin/liposome formulation can also be reduced in a lyophilized storage form. However, experiments conducted in support of the present invention show that relatively high rates of doxorubicin breakdown can occur in a lyophilized formulation under conditions of accelerated storage, even in the presence of free-radical quenchers or iron-specific chelators.

4. Summary of the Invention

It is one general object of the invention to provide a lyophilized doxorubicin/liposome composition which is stable against doxorubicin breakdown on long-term storage.

In one aspect the invention includes a lyophilized doxorubicin/liposome (L-DOX) composition which is characterized by less than 15%, and preferably less than about 10%, doxorubicin breakdown after accelerated storage in lyophilized form at 40°C for 4 weeks. The composition is prepared by lyophilizing an aqueous liposome suspension having a pH between 3.0 and 4.4, and preferably between 3.5 and 4.0, and containing (i) liposomes whose dominant lipid components are neutral phospholipids, cholesterol, and a negatively charged lipid, (ii) doxorubicin, at a drug:lipid ratio of between 5-10 percent by weight, and a doxorubicin concentration of less than 10 mg/ml, and (iii) a bulking agent.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 is an HPLC chromatogram showing doxorubicin and breakdown products in a lyophilized L-DOX composition of the invention after 2 weeks at 50°C;

5 Figure 2 is an HPLC chromatogram showing doxorubicin and breakdown products in the same L-DOX composition, but after storage in liquid suspension form for 2 weeks at 40°C; and

10 Figure 3 is an HPLC chromatogram showing doxorubicin and breakdown products in a lyophilized L-DOX composition at pH 4.8 in the presence of succinate after 2 weeks at 50°C.

Detailed Description of the Invention

A. Preparation of Lyophilized L-DOX Composition

15 Liposomes produced by the method of the present invention are formed from standard vesicle-forming lipids, typically including neutral phospholipids, such as phosphatidylcholine (PC), negatively charged lipids, such as phosphatidylglycerol (PG), phosphatidylserine (PS),
20 phosphatidylinositol (PI), phosphatidic acid (PA) or negatively charged sterol lipids, such as cholesterol sulfate and cholesterol hemisuccinate, and cholesterol or neutral cholesterol analogs. One preferred formulation includes 40-60 mole percent PC, 10-30 mole percent of a
25 negatively charged lipid, such as PG or cholesterol sulfate, and remainder cholesterol.

The phospholipid components may contain either saturated acyl chains, such as dipalmitoyl acyl chains, or unsaturated acyl chains, such as the mixture of unsaturated
30 chains present in egg PC or egg PG. The effect of acyl chain composition, lipid purity, and negatively charged lipid on storage properties, under accelerated storage conditions, is discussed in Section B below.

The liposomal lipids may also include a lipophilic free-radical quencher, such as alpha-tocopherol (α -T), alpha-tocopherol succinate (α -TS), or butylated hydroxytoluene (BHT), at a preferred concentration of about 0.5-2 mole percent of total lipids.

One preferred lipid composition, described in Example 1, contains 47.1 mg/ml egg PC (EPC), 19.9 mg/ml egg PG (EPG), 13.4 mg/ml cholesterol, and 0.91 mg/ml alpha-tocopherol acid succinate (α TS), in prelyophilized liquid-suspension form. Another preferred composition is given in Example 2.

The L-DOX composition can be prepared by a variety of liposome-forming methods, such as have been reviewed (Szoka, 1980). In one lipid-hydration method suitable for large-scale liposome production, the lipid components are initially dissolved in a volatile non-polar solvent or solvent system, such as chloroform, or a chlorofluorocarbon solvent. FreonTM 11 or a solvent system containing FreonTM 11 and 2-5 v/v percent ethanol are well suited for dissolving lipid components for use in the present invention. The low boiling point of this solvent permits rapid, and substantially complete solvent removal under the solvent-removal conditions of the invention. The solvent poses minimal health and safety-hazard risks and can be readily reclaimed by condensation.

The lipid solution is dried, under vacuum, in a suitable drying vessel. For efficient large-scale liposome production, drying and rehydration steps are preferably carried out in a planetary mixer partially filled with chemical-inert, preferably hydrophobic, spherical particles which become coated with the lipid during dehydration, as has been detailed in co-owned U.S. patent application for "Large-Scale Liposome Production," filed March 30, 1990, Serial No. 502,222. One suitable particle is a 5/16 inch

Teflon bead, preferably having a roughened surface, such as obtained from Clifton Plastics (Clifton Heights, PA). The quantity of particles added to the vessels is preferably such as to produce an area of the particles of between
5 about 0.02 and 0.04, and preferably about 0.025 and 0.03 $\text{cm}^2/\mu\text{mol}$ total lipid added to the vessel.

Planetary mixers having mixer volumes between about 1-10 liters and greater are commercially available. One preferred mixer is a 2- or 4- gallon mixer supplied by Charles
10 Ross and Sons (Hauppauge, NY). Suitable mixing speeds are given in Example 1. Drying with mixing is typically carried out for 3-4 hours for large lipid-solution volumes. After complete solvent removal, the particles in the mixer are coated with an irregular film of lipid, providing a
15 high surface area of dried lipids.

After solvent removal, the lipids are hydrated with an aqueous volume to a final concentration of lipids of between about 100-300 $\mu\text{mol}/\text{ml}$. The aqueous medium contains doxorubicin, at a concentration of between about 10-20
20 mg/ml , and preferably about 15-16 mg/ml doxorubicin in a pyrogen-free aqueous medium. Typically, the mole concentration ratio of drug to phospholipid is between 1:10 and 1:50, depending on the drug being encapsulated. For example, common doxorubicin/phospholipid concentration
25 ratios are on the order of 1:15.

Other components in the hydration medium may include desferioxamine, at a concentration of about 50 μM to 1 mM and preferably about 0.2 mM , and a physiological salt, such as NaCl, at a final concentration which gives a substan-
30 tially isoosmolar solution. The hydration medium may include a bulking agent (Section B), at a weight concentration between about 1-10%, and preferably about 5%. Alternatively, the bulking agent may be added to the re-

hydrated liposome suspension just prior to lyophilization, as described in Section B.

According to an important feature of the invention, the pH of the aqueous medium is between about 3.0 and 4.4, and preferably between about 3.5-4.0. As will be seen in Section C below, a pH of 4.4 or below significantly enhances the stability of doxorubicin against breakdown under accelerated storage conditions in lyophilized L-DOX form. At the same time, maintaining the pH above 3.0 reduces the extent of phospholipid hydrolysis, which is promoted by low pH.

One exemplary aqueous medium, used for producing doxorubicin liposomes, is prepared by dissolving desferal in pyrogen-free water, then adding doxorubicin with stirring until the drug is dissolved. To this solution is added the bulking agent and NaCl solution, to a final concentration of components of 200 μ M desferal, 5 mg/ml doxorubicin, 0.45 % NaCl, and 5% (w/v) bulking agent. The solution is adjusted to pH 3.8 by addition of HCl (Example 1).

Hydration of the lipid-coated particles preferably occurs under mixing conditions similar to those used in preparing the lipid-coated particles. In particular, for large-scale preparation, the hydration procedure is preferably carried out in a planetary mixer, under the mixing condition described above. The thorough mixing action provided by the bi-axial motion of the mixing blades breaks up particle-particle aggregates, and thus exposes more lipid-coated surface area for hydration. The greater degree of lipid surface exposure to the aqueous medium enhances the rate and final yield of liposome formation.

The liposome suspension may be sized to achieve a selected size distribution of vesicles in a size range less than about 1 micron and preferably between about 0.05 to 0.5 microns, and most preferably between about 0.05 and 0.2

microns. The sizing serves to eliminate larger liposomes and to produce a defined size range having optimal pharmacokinetic properties.

Several techniques are available for reducing the sizes and size heterogeneity of liposomes. Extrusion of liposomes through a small-pore polycarbonate membrane is an effective method for reducing liposome sizes down to a well-defined size distribution whose average is in the range between about 0.08 and 1 micron, depending on the pore size of the membrane. Typically, the suspension is cycled through the membrane several times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

Free doxorubicin, i.e., doxorubicin present in the bulk aqueous phase of the medium, is preferably removed to increase the ratio of liposome-entrapped to free drug. The drug removal is designed to reduce the final concentration of free doxorubicin to less than about 20% and preferably, less than about 10% of the total drug present in the composition.

Several methods are available for removing free drug from a liposome suspension. A sized liposome suspension can be pelleted by high-speed centrifugation, leaving free drug and very small liposomes in the supernatant. Another method involves concentrating the suspension by ultrafiltration, then resuspending the concentrated liposomes in a drug-free replacement medium. Alternatively, gel filtration can be used to separate larger liposome particles from solute (free drug) molecules.

One preferred procedure for removing free doxorubicin, or analog thereof, utilizes an ion-exchange resin capable of binding drug in free, but not in liposome-entrapped, form. The preferred resin is a cation-exchanger, since the

drug is positively charged at neutral pH. One preferred ion exchange resin is Dowex 50W-X4 50-100 mesh resin.

After free drug removal, the liposomes may be diluted by addition of a physiological buffer, or concentrated, e.g., by ultrafiltration, to a desired drug concentration. Bulking agent, if not already present, is added to the suspension at this stage. The liposomes are then lyophilized for storage, as will now be described.

10 B. Lyophilization and Storage

In general, storage of drug/liposomes in liquid suspension form over prolonged periods can lead to loss of entrapped drug from the liposomes, liposome size changes, and degradative changes in the lipid and/or drug molecules in the composition. In general, degradative changes in a liposome or cell suspension can be arrested, for long-term storage, by freezing or lyophilization, i.e., freezing followed by water removal by sublimation under vacuum.

As is known, the steps of freezing, dehydrating, and/or rehydrating dried liposomes can themselves cause undesired changes in drug/liposome properties. Studies conducted in support of the present invention, as well as studies reported by others (e.g., Anchordoguy, 1988, 1987; Higgins, 1987, 1986; Strauss), suggest that liposomes may undergo two types of disruptive damage during freezing, prior to lyophilization. One type of damage is membrane rupture caused by ice crystal formation inside and outside the vesicle spaces during freezing. This type of damage can lead to substantial loss of an encapsulated, water-soluble drug molecule. Such drug solute loss due to membrane rupture can be reduced by cryoprotectants such as glycerol, DMSO, polyethylene glycol, polypropylene glycol, 1,3,-butanediol, 2,3, butanediol, 1,3-propanediol, a variety of mono- and disaccharides, such as lactose, sucrose, and trehalose, and polysaccharides, such as

dextran and hydroxyethyl starch (HES) which appear to interrupt or minimize ice crystal formation, when present both inside and outside the liposomes.

In addition to membrane rupture, as evidenced by a
5 loss of encapsulated solute, liposomes tend to show a progressive size growth with freeze/thaw cycles. The liposome size growth may be due to a solute-exclusion effect in which the formation of ice crystals concentrates solutes and liposomes into microenvironments of very high lipid and
10 salt concentrations, in effect, forcing liposome together. This effect can be reduced by including in the bulk (extra-liposomal) phase of a liposome suspension, a bulking agent which is effective to interrupt formation of large ice crystals.

15 The bulking agent should also have the property of forming a solid, somewhat porous, non-crystalline matrix on drying, to allow escape of water by sublimation from the frozen sample, and to reduce additional solvent exclusion effects due to crystal formation in the bulking agent.
20 Note that the requirement for a solid bulking agent excludes a variety of small viscous-liquid cryoprotectants such as DMSO, glycerol, ethylene glycol, and propylene glycol as the bulking agent in the present invention.

One general class of compounds which are suitable as
25 bulking agents are non-crystalline carbohydrates, such as trehalose and lactose, and polysaccharides, such as dextrans and hydroxyethylcellulose. The use of these compounds as cryoprotectants or bulking agents for liposomes, microsomal membranes or blood cells has been
30 reported (e.g., Crowe; Anchordoguy, 1988; Strauss; Madden).

Another general class of bulking agents includes amino acids, and amino acid analogs, such as 2-aminobutyric acid, 4-hydroxyproline, sarcosine, glycine betaine, and basic
35 amino acids, such as lysine and histidine, which may inter-

act with the negatively charged head groups of the liposomes (Anchordoguy, 1988).

5 A third general class of bulking agents includes a variety of non-sugar glycolytic pathway compounds, such as sodium or potassium salts of tartrate, oxaloacetate, fumarate, malate, ketoglutarate, and pyruvate. The bulking agent is preferably a mixture of two or more of these compounds, to minimize crystal formation effects on freezing and dehydration. As will be seen in Section C below, succinate is not a suitable bulking agent since it enhances doxorubicin breakdown on storage in lyophilized form, nor are di- or tri-dicarboxylic acid compounds, such as citrate, since these may precipitate with doxorubicin, which is positively charged. A fourth class of bulking agents includes non-saccharide polymeric compounds, such as higher molecular weight polyethylene glycol (PEG), which are (a) solids at room temperature, (b) readily soluble in water, and (b) pharmaceutically acceptable for parenteral administration. In particular, PEG polymers with molecular weights above about 1,500-2,000 daltons are contemplated. In one embodiment, the polymer is included in the bulk phase of the suspension, at a weight concentration between about 1-10 percent.

25 In another embodiment, the liposomes themselves are derivatized with a PEG or other polyalkyl oxide, for purposes of enhanced lifetime in the bloodstream when administered intravenously, as described in co-owned U.S. patent application for "Liposomes with Enhanced Circulation Times," filed October 10, 1989, Serial No. 425,224. Here the surface-derivatized molecules act to prevent ice crystal growth in the region of the liposomes. The liposome suspension is preferably concentrated to insure a relatively high polymer concentration in the bulk phase. The liposome suspension may also contain a solution-phase

bulking agent selected from one of the classes above, in addition to the liposome-bound agent.

The bulking agents may be included in the aqueous hydration medium used in forming the liposomes, yielding a suspension with an equal concentration of agent in the bulk and encapsulated liposomal aqueous phases. Alternatively, the agent can be added to the final sized liposome suspension, wherein the agent is present only in the bulk phase of the suspension. As indicated above, the final concentration of the bulking agent in the suspension is between about 1-10 weight percent and preferably about 4-6 weight percent.

In still another embodiment, liposome fusion on freezing and dehydration is minimized by forming the initial liposome dispersion in a low-ionic-strength medium in which the doxorubicin/liposomes have a partially ordered gel structure, by virtue of the surface charge repulsion effects. This type of liposome gel has been described in co-owned U.S. application for "Liposome Gel Composition and Method," filed May 22, 1989, Serial No.356,262. The gel formulation requires an ionic strength comparable to that of NaCl, at a concentration of less than about 20 mM NaCl. The medium may contain non-ionic species, such as mono or disaccharide protective agents.

In forming the lyophilized freeze-dried suspension, the liposome dispersion is frozen and lyophilized as described below. The lyophilized material can be reconstituted, for parenteral injection, by addition of a

rehydration medium containing physiological salts or other ionic species.

In a preferred lyophilization method, the suspension is first cooled to 4-5°C, then frozen to -45°C and maintained at this temperature for 12 hours in a conventional lyophilizer. The lyophilizer chamber is pumped down to about 200 μ pressure, after which the chamber temperature is raised, at about 10°C/hr, to -20°C. The chamber is maintained at this temperature until the lowest reading product thermocouple is within 3°C of shelf temperature.

The chamber temperature is now allowed to rise, again at about 10°C/hour, to room temperature, and the material is held under vacuum at this temperature for an additional 15 hours. The chamber is backfilled with nitrogen to a pressure of about 2 inches Hg, and the sample vessels stoppered for storage. One suitable lyophilizer is a Edwards Lyoflex 08 lyophilizer supplied commercially from Edwards High Vacuum, Inc. (New York).

The stability of the lyophilized preparation can be examined by accelerated studies conducted at elevated temperatures, according to known principles. The storage conditions which were used in the studies described below were 40°C for 4 weeks, and 50°C for two weeks. In each storage study, the lyophilized sample in a stoppered vessel was placed in an incubator at the selected temperature and analyzed after the two- or four-week incubation period for doxorubicin breakdown, with the results discussed in Section C.

C. Doxorubicin Stability on Storage

Table I below shows composition and pH variables in several lyophilized L-DOX preparations which were studied under accelerated storage conditions. Each of the 20 preparations were prepared according to the methods outlined above, and detailed for Preparation No. 11 in Example 1.

TABLE 1

	<u>NO.</u>	<u>Anionic Lipid</u>	<u>Anti-Oxidant</u>	<u>Desferal</u>	<u>Succinate</u>	<u>pH</u>
5	1	EPG	σ TS	.2	+	4.8
	2 ^a	EPG	σ TS	.2	+	4.8
	3	EPG	σ TS	.2	-	4.8
	4	EPG	-	.2	+	4.8
10	5	EPG	BHT	.2	+	4.8
	6 ^b	DPPG	σ TS	.2	+	4.8
	7	EPG	σ TS	-	+	4.8
	8	EPG	σ TS	1.0	+	4.8
	9 ^c	EPG	σ TS	.2	+	4.8
15	10 ^d	CS	σ TS	.2	+	4.8
	11	EPG	σ TS	.2	-	3.8
	12	EPG	σ TS	.2	-	3.0
	13 ^e	EPG	σ TS	.2	-	3.8
	14	EPG	σ TS	.2	+	3.8
20	15	EPG	BHT	.2	-	4.0
	16	EPG	BHT	.2	-	4.2
	17	EPG	σ T	.2	-	3.8
	18	-	-	-	-	5.3
	19	-	-	.2	+	4.8
25	20	-	-	.2	-	5.3

^a99% pure EPC and EPG from Avanti Polar Lipids, Inc. (Birmingham, AL) were used.

30 ^bA 99% pure dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) (Avanti Polar Lipids) were used to assess the effect of the degree of fatty acid saturation on the stability profile.

35 ^c99% pure EPG from Genzyme Corp. (Boston, MA) was utilized.

40 ^dEPG was completely removed from the formulation; sodium cholesterol sulfate was incorporated in its place to provide the negative charge for the optimal incorporation of doxorubicin into liposomes.

45 ^e10mM lactic acid substituted for 10 mM succinic acid in the aqueous phase.

After storage under the above accelerated storage conditions, each preparation was reconstituted by addition of distilled water to a final doxorubicin concentration of about 5 mg/ml. An aliquot of the material was diluted with mobile phase to a final dilution of 1:50 at a final liquid concentra-

tion of about 2.5 $\mu\text{mol/ml}$ (1.75 mg/ml). At this concentration, the lipid and drug components of the L-DOX suspension are in solution. A typical example of this material, chromatographed by HPLC is detailed in Example 3.

5 Figure 1 shows an HPLC profile of doxorubicin and doxorubicin breakdown products observed in a lyophilized preparation corresponding to composition No. 11 in Table 1, after accelerated storage for 2 weeks at 50°C. The peaks in the chromatogram represent absorbance at the doxorubicin absorbance peak
10 of at 480 nm. Three distinct peaks, with RT (retention time) values of 9.92, 12.75, and 14.4 (doxorubicin) were observed, with the measured relative peak areas shown. The vertical markers on either side of each peak indicate the portion of the curve that was integrated.

15

TABLE 2

	<u>Peak#</u>	<u>Area%</u>	<u>RT</u>
	1	0.695	9.92
20	2	1.227	12.75
	3	98.077	14.4

The concentration of doxorubicin in the reconstituted sample, both before and after storage at 50°C for two weeks,
25 was determined from a standard curve of known concentrations of doxorubicin as a function of peak areas on HPLC. The values calculated were 4.96 ± 0.03 and 4.51 ± 0.05 mg/ml doxorubicin before and after storage (Table 5). Thus, about 9% of the total doxorubicin present in the pre-stored sample was lost to
30 breakdown products on storage.

The doxorubicin peak in the Figure 1 HPLC profile constitutes all but about 2% of the detected products, leaving approximately 7% of the breakdown products unaccounted for. One possible source of this discrepancy is that one or more of
35 the breakdown products have lower absorbance coefficients at 480 nm, so that these products are either not seen or underes-

timated in amount. Another possible source of discrepancy is that the breakdown products do not have well-defined peaks, and therefore are not included in the total peak area calculations. Finally, it is possible that some of the breakdown products are not eluted from the column under the chromatography conditions employed.

Figure 2 shows an HPLC profile of the same L-DOX formulation as in Figure 1, but after accelerated storage at 40°C for two weeks in liquid suspension form. The relative peak areas of the four peaks are given in Table 3 below. The total amount of doxorubicin in the main peak in the figure represents about 67% of the doxorubicin present prior to storage. Thus, storage in liquid suspension form substantially increases the breakdown of doxorubicin in the a low-pH L-DOX formulation.

TABLE 3

	<u>Peak#</u>	<u>Area%</u>	<u>RT</u>
20	1	5.193	10.16
	2	87.212	14.88
	3	4.808	20.06
	4	2.407	21.15

Figure 3 shows an HPLC profile of a lyophilized L-DOX formulation corresponding to composition No. 2 in Table 1, after accelerated storage at 50°C for 2 weeks. As seen in the Figure, and in Table 4 below, at least 16 different peaks were identified, with the doxorubicin peak accounting for about 85% of the total peak area. The total amount of doxorubicin in the main peak in the figure represents about 74% of the doxorubicin present prior to storage.

TABLE 4

	<u>Peak#</u>	<u>Area%</u>	<u>RT</u>
5	1	0.069	3.34
	2	0.104	3.6
	3	0.129	4.03
	4	0.216	7.35
10	5	0.427	9.25
	6	0.729	10.38
	7	0.077	11.11
	8	0.705	13.63
	9	85.509	15.31
15	10	0.344	20.75
	11	0.623	21.58
	12	0.631	22.31
	13	0.124	25.45
	14	0.302	30.48
20	15	0.756	31.55
	16	1.255	69.61

Each of the 20 formulations in Table 1 were tested as above, for loss of doxorubicin after storage at 40°C or 50°C for 4 or 2 weeks, respectively. The results are given in Table 5 below, along with the pH for the suspension, prior to lyophilization.

TABLE 5

5	No.	Formulation Description	Formulation pH ^c	[DOX] ^a mg/mL (% of Original)		
				Original	2 weeks at 50°C ^b	4 weeks at 40°C ^b
10	1		5.2	4.92±0.07	2.44±0.04 (50%)	3.99±0.03 (81%)
	2	high purity EPC & EPG	5.2	4.90±0.04	2.13±0.09 (44%)	3.58±0.07 (73%)
	3	No Succinate	6.0	4.86±0.04	3.41±0.07 (70%)	4.08±0.01 (84%)
15	4	No σ Ts	5.3	4.90±0.02	2.64±0.09 (54%)	3.94±0.10 (80%)
	5	BHT, no σ Ts	5.3	5.05±0.23	2.48±0.25 (49%)	3.94±0.08 (78%)
20	6	DPFC/DPPG ^c	5.0	4.32	1.69 (39%)	3.72 (86%)
	7	No Desferal	5.0	4.76±0.06	3.21±0.07 (67%)	4.05±0.06 (85%)
	8	1 mM Desferal	5.1	4.68±0.16	2.32±0.88 (50%)	4.10±0.02 (88%)
25	9	high purity EPG	5.1	4.69±0.06	3.08±0.20 (66%)	3.92±0.04 (84%)
	10	Chol. Sulphate, No EPG	5.2	4.92±0.11	3.51±0.02 (71%)	4.28±0.00 (87%)
	11	No Succinate, pH 3.8	3.8	4.96±0.03	4.51±0.05 (91%)	4.58±0.07 (92%)
30	12	No Succinate, pH 3.0	3.2	4.75±0.03	4.50±0.03 (95%)	4.51±0.02 (95%)
	13	Lactate, No Succinate	3.8	4.73±0.02	3.88±0.12 (82%)	4.31±0.03 (91%)
35	14	Succinate, pH 3.8	3.9	5.08±0.00	3.61±0.09 (71%)	4.26±0.02 (84%)
	15	No Succinate, pH 4.0	3.9	5.03±0.05	4.41±0.04 (88%)	* ^d
	16	No Succinate, pH 4.2	4.1	5.11±0.07	4.38±0.04 (86%)	* ^d
40	17	σ T, No σ Ts, No Succinate, pH 3.8	3.7	5.34±0.03	4.81±0.07 (90%)	* ^d
	18	DOX	---	5.07	5.06±0.03 (100%)	4.74±0.06 (94%)
45	19	DOX in Succinate buffer	4.8	5.25±0.22	3.64±0.09 (69%)	4.80±0.14 (91%)
	20	DOX in Buffer, No Succinate	5.1	5.49±0.05	5.15±0.04 (94%)	5.28±0.14 (96%)
50	[DOX] was measured using HPLC. Triplicate vials were analyzed at each time point, with the exception that 4 weeks, 40°C samples 2-5, 19 and 20 were done in duplicate.					
55	4.5 mL of pre-lyophilization bulk suspension (nominal [DXN] = 5.0 mg/mL) was filled into 20cc clear type I glass vials for lyophilization. Reconstitution was accomplished by adding 4.0 mL of water to provide a target [DXN] of 5.0 mg/mL. All vials were stored upright during the incubation period.					

The most important factors effecting doxorubicin stability were reduced pH (between pH 3-4) and the absence of succinate. Removal of succinic acid from the L-DOX formulation significantly increased drug stability (sample #3 vs. sample #1). Less doxorubicin degradation was also seen when the pH of the formulation was lowered to 3.8 (sample #14 vs. sample #1). Maximal stability was obtained with the simultaneous elimination of succinic acid and lowering of pH. For example, only 5% degradation was observed in a pH 3.0 formulation that did not contain succinic acid (sample #12) after two weeks incubation at 50°C., whereas a 9-10% decrease in drug potency was exhibited in similar formations at pH 3.8 under the same condition (sample #11 and sample #17). Increasing the pH further to 4.0 and 4.2 (sample #15 and sample #16) resulted in slightly more DOX degradation. Adding lactate resulted in significantly more DOX degradation (sample #13 vs. sample #11), although lactate had a less detrimental effect than succinate in this regard (sample #13 vs. sample #14). A less dramatic effect was observed in samples incubated at 40°C than those incubated at 50°C. No lysophosphatidylcholine (lyso-PC) was detected in any of these samples after incubation at 40°C or 50°C (data not shown).

No systematic trend relating the stability profile to the purity, degree of saturation of phospholipid used, or type of negatively charged lipid used was seen, based on results from samples incubated at 50° C. The use of EPG from Genzyme Corporation and sodium cholesterol sulfate

30 ° DPPC - Dipalmitoylphosphatidylcholine
DPPG - dipalmitoylphosphatidylglycerol
50 mL batch size was prepared for this formulation; only one vial was analyzed for each time point.

35 ° Data are not yet available at this time.

° pH of the prelyophilization bulk suspension except for samples 6-10 (pH of reconstituted samples).

appears to result in some improvement in DOX potency preservation compared to the original formulation (sample #1) at the end of the incubation period. No dramatic effect was observed in samples incubated at 40°C.

- 5 To assess the effect of the antioxidant on the stability profile, α -Ts was removed (sample #4) and replaced with BHT (sample #5). No significant difference in DOX stability was observed in these samples at either 50°C or 40°.

- 10 Doxorubicin stability was compared in formulations containing no Desferal (sample #7), 200 μ M (sample #1) or 1 mM Desferal (sample #8). The samples without Desferal, incubated at 50°C, showed a slight improvement in DOX stability.

- 15 The present invention is useful as an improved storable form of doxorubicin/liposomes, for use in the treatment of a variety of tumor types. Reduced side effects have been observed in Phase I and Phase II clinical trials with doxorubicin liposome formulations prepared by reconstituting lyophilized preparations. The present L-DOX invention provides the additional advantage of long-term stability on storage, without significant loss of active drug or generation of undesired breakdown products. The preparation is easily prepared by a method that is suitable for large-scale manufacture.

- 25 It will be appreciated that the advantages and features of the present invention will apply to a variety of related anthracene glycoside anti-neoplastic drugs, such as daunomycin, carinomycin, N-acetyladrinamycin, N-acetyl-daunomycin, rubidazole, 5-imidodaunomycin, and epirubicin.

- 30 The following example illustrates the method of the invention for preparing doxorubicin liposomes. The example illustrates, but in no way is intended to limit the scope of the invention.

Example 1Preparation of Lyophilized L-DOX Composition(Preparation 11, from Table 1)

A 300 ml chloroform solution containing 20.1 g EPC
5 (Lipoid), 8.5 g EPG (Asahi), 5.7 g CH (Croda), and 0.4 g α -
tocopherol succinate (Henkel) was added to a 2-liter round-
bottomed flask containing 180 g of 3 mm diameter glass beads
and the whole dried in vacuo using a rotary evaporator. The
lipid film was subsequently exposed to a vacuum of 50 mTorr
10 overnight to complete drying.

The lipids were hydrated to a final total lipid concen-
tration of about 240 μ mole/ml by addition of a doxorubicin-
containing solution and mechanically agitating the mixture for
2 hours at room temperature. This solution contained about 15
15 mg/ml DOX, 5% (w/v) lactose monohydrate, 0.4% (w/v) sodium
chloride, 200 μ M desferal in water and was prepared by dissol-
ving the doxorubicin in water and adding the other excipients
when complete dissolution was achieved. The pH of the mixture
was adjusted to 3.8 with HCl. The resultant liposome suspen-
20 sion was sized by passage five times through 0.4 μ m and five
times through 0.2 μ m pore-size polycarbonate membranes.
Unincorporated DOX was removed by passing the sized liposome
suspension over a Dowex 50W-X4 cation exchange resin.
Finally, the suspension was diluted to a DOX concentration of
25 about 5 mg/ml prior to lyophilization, using a 5% lactose,
0.4% sodium chloride, 200 μ M desferal solution, pH 3.8. The
characteristics of this pre-lyophilization solution are given
in Table 6.

TABLE 6

	<u>Theoretical Composition</u>	<u>mg/ml</u>
5	DOX HCl	5.0
	EPC	47.1
	EPG	19.9
	CH	13.3
	α -TS	0.91
10	Desferoxamine mesylate	0.132
	Lactose monohydrate	52.6
	NaCl	4.0
	Water for injection	qs to 1 ml
	HCl	qs to pH 3.80
15	Assay values for pre-lyophilization solution:	
	Doxorubicin HCl (mg/ml)	4.91
	% encapsulation	96
20	Liposome diameter (nm)	290

Example 2Large-Scale L-DOX Production

25

A lipid solution containing 1500 ml Freon 11TM, 151.1 g EPC (Lipoid), 63.9 g EPG (Asahi), 42.9 g cholesterol (Croda), and 0.86 g of butylated hydroxytoluene (Penta) was added to a 2-gallon double planetary mixer (Ross model 130 LDM) which contained about 4,100 PTFE Teflon beads (Clifton Plastics) having a mean diameter of 1/4 inch. The beads had been washed in an aqueous solution containing a detergent (Alconox) to remove any oily residues from the surfaces of the beads, rinsed with ethanol, and dried, before placement into the mixer.

The mixer was sealed and the pressure inside the mixer was reduced while the solution and beads were mixed at an orbital stirring rate of 20 rpm and an axial stirring rate of 26 rpm for about two hours, to transform the lipids to a solid state attached to the beads. A vacuum was maintained for an additional 3 hours without mixing to achieve additional solvent removal.

After solvent removal, an aqueous solution of doxorubicin HCl to a final lipid concentration of about 262 μ mol/ml was added to the lipid-coated particles in the planetary mixer. The drug solution was prepared to contain 0.132 mg/ml deferoxamine mesylate in pyrogen-free distilled water. Doxorubicin hydrochloride (Farmitalia Carlo Erba) was then added with stirring to a final concentration of about 12.5 mg/ml. After complete dissolution of the drug, lactose was added to a final concentration of 5 weight percent and NaCl 0.4 weight percent. The pH of the solution was then adjusted to 3.7 with HCl solution.

Hydration of the lipid was carried out at a temperature of 25° C. for 2 hours, with mixer stirring at about 25 rpm. The resulting liposome suspension was sized by passes through 0.4 and 0.2 micron polycarbonate filters. Free doxorubicin was reduced by treating the sized-liposome suspension with a Biorad AG 50W-X4 50-100 ion exchange resin. The preparation was then diluted with a solution containing deferoxamine mesylate, lactose, sodium chloride and hydrochloric acid in pyrogen-free distilled water.

The approximate final composition of the liposome suspension was:

	4.3 mg/ml doxorubicin hydrochloride
	40.0 mg/ml EPC
25	16.9 mg/ml EPG
	11.3 mg/ml cholesterol
	0.23 mg/ml butylated hydroxytoluene
	0.132 mg/ml deferoxamine mesylate
	50.0 mg/ml lactose
30	4.0 mg/ml sodium chloride

The preparation had the following properties: pH of approximately 3.8, mean particle size of approximately 232 nm; approximately 96% of the total drug was liposome associated.

A. HPLC Chromatography

Lyophilized L-DOX was reconstituted with water to a final doxorubicin concentration of about 5 mg/ml. A 0.25 ml aliquot of the rehydrated suspension was diluted to 5 ml with mobile
5 phase.

The separation of the doxorubicin and its degradation products was carried out on a Waters HPLC using a Whatman Partisil ODS-3 column, 250 x 4.6 mm held at ambient temperature. The mobile phase was 43% aqueous buffer in methanol.
10 The aqueous buffer was 95 mM ammonium phosphate/ 5 mM triethylamine, pH 4.0. 15 µl of sample was injected in the system at a 1 ml/min flow rate. Effluent was monitored at 480 nm. An external standard, prepared fresh each day, consisted of doxorubicin powder dissolved in mobile phase to a final
15 concentration of 0.35 mg/ml.

B. Analysis after Storage

After an incubation period of two weeks at 50°C, the L-DOX was analysed by HPLC, as above. Doxorubicin potency was
20 reduced 9%. The level of doxorubicin breakdown appeared to plateau at this level: a further two-week incubation at 40°C did not result in further reduction of doxorubicin concentration.

25 Although preferred methods and formulations have been

25

described, it will be apparent that various changes and modifications may be made without departing from the invention.

IT IS CLAIMED:

1. A lyophilized doxorubicin/liposome composition which is:

5 (a) characterized by less than 15% doxorubicin breakdown after storage in lyophilized form at 40°C for 4 weeks, and

(b) prepared by lyophilizing an aqueous liposome suspension having a pH between 3.0 and 4.4 and containing (i) liposomes whose dominant lipid components are neutral phospholipids, cholesterol, and a negatively charged lipid, (ii) doxorubicin, at a drug:lipid ratio of between 5-10 mole percent, and a doxorubicin concentration of less than 10 mg/ml, and (iii) a cryoprotectant.

15 2. The composition of claim 1, which is characterized by less than 10% doxorubicin breakdown after such storage, and the suspension pH is between 3.5 and 4.0.

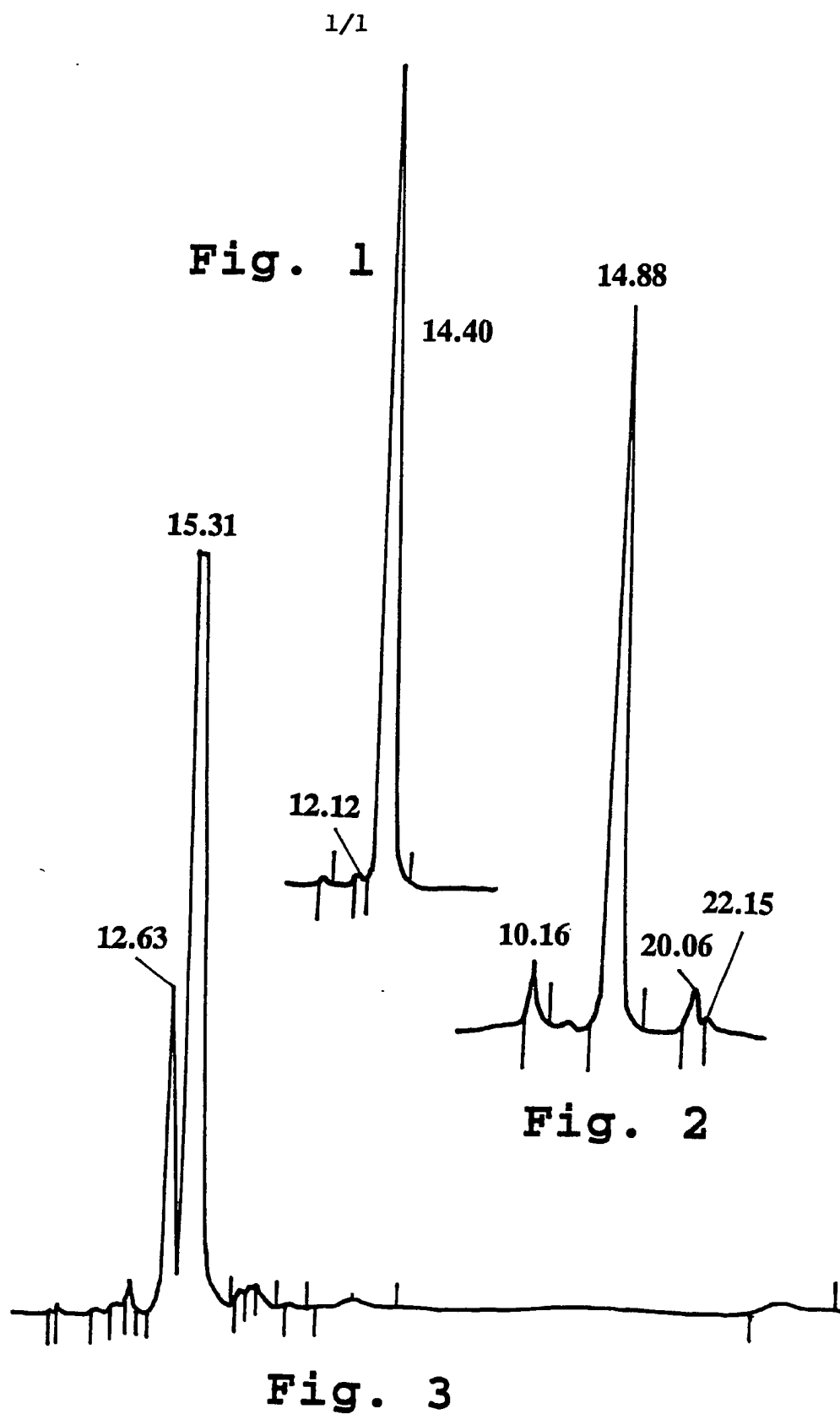
3. The composition of claim 1, which is substantially free of succinate.

4. The composition of claim 1, wherein the liposomes contain between about 40-60 mole percent phosphatidylcholine, 20-40 mole percent cholesterol, and 10-30 mole percent phosphatidylglycerol.

5. The composition of claim 1, wherein the liposomes contain a lipophilic free radical quencher selected from the group consisting of alpha-tocopherol or acid or salt thereof, and butylated hydroxytoluene.

6. The composition of claim 1, wherein doxorubicin is present in the suspension at a concentration between about 4-6 mg/ml.

7. A method of storing doxorubicin in a stable, liposome-entrapped form, as evidenced by less than 15% doxorubicin breakdown after storage at 40°C for 4 weeks, comprising lyophilizing an aqueous liposome suspension having a pH
5 between 3.0 and 4.4 and containing (i) liposomes whose dominant lipid components are neutral phospholipids, cholesterol, and a negatively charged lipid, (ii) doxorubicin, at a drug:lipid ratio of between 5-10 mole percent, and a doxorubicin concentration of less than 10 mg/ml, and (iii) a cryo-
10 protectant, and storing the lyophilized suspension.
8. The method of claim 7, which is effective to produce less than 10% conversion of doxorubicin to inactive degradation products after such accelerated storage, and the suspension pH is between 3.5 and 4.0.
15
9. The method of claim 7, wherein the liposomes contain between about 40-60 mole percent phosphatidylcholine, 20-40 mole percent cholesterol, and 10-30 mole percent phosphatidylglycerol.
20
10. The method of claim 7, wherein the liposomes contain a lipophilic free radical quencher selected from the group consisting of alpha-tocopherol or acid or salt thereof, and
25 butylated hydroxytoluene.
11. The composition of claim 7, wherein doxorubicin is present in the suspension at a concentration between about 4-6 mg/ml.



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/05519

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 A61K9/127		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	WO,A,8 809 168 (LIPOSOME TECHNOLOGY, INC) 1 December 1988 ---	1-11
Y	JOURNAL OF CONTROLLED RELEASE. vol. 9, no. 3, August 1989, AMSTERDAM NL pages 215 - 229; G.STORM ET AL.: 'LIPOSOME ENCAPSULATION OF DOXORUBICIN' ---	1-11
<p>⁹ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
14 NOVEMBER 1991	21. 11. 91	
International Searching Authority	Signature of Authorized Officer	
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